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GC-MS profile, antioxidant, analgesic, and anti-inflammatory potential of essential oil of *Salix alba*

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Abstract
This work aims to evaluate the antioxidant, analgesic, and anti-inflammatory activity of the essential oil of *Salix alba*. First, the essential oil of *Salix alba* was extracted using the hydrodistillation method, and GC-MS was used to determine their chemical composition. Then, the antioxidant activity was conducted using the DPPH radical scavenging method. The analgesic action was tested by the hot plate and writhing test. On the other hand, the anti-inflammatory activity was determined using the formalin test. Thirty-one compounds were identified. The main components were identified as 3-Methylpentane (4.57%), 1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane (Eucalyptol) (2.93%), E-2-methylbut-2-enal (2.50%), and butyl 2-butoxy-2-hydroxyacetate (1.48%). The results showed that essential oil has interesting antiradical properties. It was manifested by a low value of IC50 (0.017 mg/ml). The analgesic effect detected some temperature resistance with a 6 sec against Tramadol (3.33 $\pm$ 0.6 sec). Thus, it reduced the pain caused by the impact of acetic acid with a writhing number equal to 7.66 $\pm$ 2.33. The study of the anti-inflammatory activity in vivo showed that the essential oil of *S. alba* reduced the inflammatory reaction in the induction model by formalin. From these results, it can be concluded that the essential oil of *Salix alba* exhibits essential antioxidant, analgesic, and anti-inflammatory activities.

Keywords
Extraction, *Salix alba*, GC-MS, Analgesic, Inflammation, Essential oil

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Contents

1. Introduction

Herbal remedies have been used in herbal medicine for human diseases because of their richness in hundreds or even thousands of components of therapeutic interest [1]. Indeed, plants contained chemical components that were divided into groups: proteins, carbohydrates, lipids, and nucleic acids on the one hand, pigments, polyphenols, polymers, hormones, and volatile compounds (Essential Oils, E.O.) on the other hand [2]. Essential oils contain alkaloids and phenols, part of this group of secondary metabolisms. These essential oils were named after plant essences, aromatic essences, volatile oils, or perfumes [3,4]. The pharmacological properties of essential oils have been widely studied [5]. Oxidative stress was involved in many diseases as a triggering factor or associated with severe complications. Most oxidative stress-induced diseases appear with age as aging decreases antioxidant defenses, increasing mitochondrial multiplication of radicals [6]. Among the biological activities of essential oils, attention has been focused on antioxidant activity because of their role in preventing chronic diseases such as heart disease,

cancer, diabetes, hypertension, and Alzheimer's disease in combating oxidative stress and faced with the development of in vivo research and the therapeutic limits of chemical drugs against the inflammation and toxicity of synthetic antioxidants, research on medicinal plants has been oriented towards obtaining natural substances with anti-inflammatory and antioxidant activities [7,8]. White willow (*Salix alba L.*), belonging to the genus Salix and Salicaceae family, has a long history of medicinal usage, dating back to nearly 6000 years. It is found in all temperate and humid zones in Europe, North Africa, and Central Asia [9]. The bark and leaf of willow species contain the salicin prodrug, identified in 1829 by the French pharmacist H. Leroux. The antipyretic properties described in all the publications confirm the febrifugal action of the Willow extract [10]. The present work aimed to evaluate *in vitro* antioxidant activity and the in vivo analgesic and anti-inflammatory activity of the essential oil extracted from *Salix alba* after determining the essential oil's chemical composition.

2. Materials and Methods

2.1 Collection of samples

Leaves and flowers of *S. alba* were harvested in February 2018. The plant was identified by botanists at the Department of Biology of the University of Mustapha Stambouli, Mascara, Algeria.

2.2 Experimental animals

The model chosen was the albino rat Wistar *"Rattus norvegicus"* with an average body weight of 150-200 g, provided by the University of Mustapha Stambouli, Mascara, Algeria laboratory. The rats were divided into groups and were kept at a temperature of 20 ± 1 °C with a natural photoperiodic cycle. They were fed with food pellets and water *ad-libitum*. All experiments were approved by the Animal Ethics Committee of the Mustapha Stambouli University, and the procedures used during these studies were according to the European directive concerning animal testing [11].

2.3 Extraction of essential oil

Hydrodistillation was used to extract the essential oils since it was the standard method for the essential oil extraction with the best yield. 100 g of aerial parts of *S. alba* was boiled at 100°C. When the temperature stabilized, the distillate was collected. Then, the mixture was separated using cyclohexane, and the organic fraction was taken to undergo rotary evaporation to remove the solvent and obtain the essential oil. The essential oil was stored at +4 °C after the yield calculation.

2.4 GC-MS analysis

GC-MS analysis was performed using a chromatograph TQ8030 series equipped with an FID detector in a split injector mode and a silica column (30 m × 0.25mm and film thickness 0.25 μm). Oven temperature programming was 50-230 °C with an increase of 5°C/min. The injector temperature was 230°C. The carrier gas was helium and flew at a rate of 4.38 ml/min with linear Velocity mode and pressure of 96.2 kPa. Identification was based on sample retention data, a comparison with authentic standards, and computer matching using the NIST MS library. The identification was also confirmed by comparison of the retention indices with data in the literature. The percentages of compounds were calculated using the area normalization method without considering response factors. The retention indices were calculated for all volatile constituents using a homologous series of n-alkanes.

2.5 DPPH free radical scavenging assay

The antiradical activity of *S. alba* E.O. was determined according to the method of [12], which used DPPH as a relatively free radical that absorbed in the visible at the wavelength λ= 517 nm. The DPPH solution was prepared by solubilizing 2.4 mg of DPPH in 100 ml of absolute methanol. 25 μl of the E.O. at different concentrations were added to 975 μl of DPPH solution. A standard antioxidant solution (ascorbic acid) was also prepared under the same conditions to be a positive control. The negative control consisted of DPPH and methanol. The mixture was left in the dark for 45 minutes. The assay was performed spectrophotometrically at a wavelength of 517 nm. The percentage of antiradical activity was estimated according to the equation:

$$Anti-radicalactivity\% = [\frac{(A1-A2)}{A1}] \times 100 \quad (1)$$

A1: Absorbance of the negative control without extract

A2: Absorbance in the presence of the extract.

Inhibitory Concentration 50 (IC_{50}) is the test sample concentration required to reduce 50% of the DPPH radical. The IC50s are calculated graphically by inhibition percentage based on different concentrations of the extracts. For the entire experiment, each test is performed in triplicate.

2.6 Analgesic activity (hot plate method)

The heating plate test was performed according to the method described by [13]. The test was based on the use of 09 rats divided into three groups: the first group was used for the control treated with saline solution, and the second was treated with essential oil of *S. alba* (250 μl / kg) given orally. The last group was used for the standard treated with Tramadol (250 μl/ kg) given orally. After administration of the treatments, the rats of each group were placed on the heating plate: [50-55 °C]. The reaction time indicated the animal's resistance to pain as a latency time and was used to evaluate the effectiveness

of analgesic substances. Then, the reaction time was measured after 30 min, 60 min and 90 min. Mean latency was determined from the three rats in each group.

2.7 Analgesic activity (writhing test)

The writhing test was a chemical method described by [14]. This method was used to induce peripheral pain by injecting irritating reactive such as acetic acid. 09 rats were divided into three groups: the first was used for the control treated with distilled water, and the second was treated with essential oil of *S. alba* (250 μl / kg) given orally. The last group was treated with Paracetamol (250 μl / kg) orally at a rate of 10 ml/kg. 30 minutes after the treatments, the rats intraperitoneally received 1.2% acetic acid at a rate of 10 ml/kg. After five minutes, the number of contortions was counted for each rat for 10 minutes.

2.8 Anti-inflammatory activity (the formalin test)

The anti-inflammatory effect was evaluated based on the induction method of edema by injecting formalin in the paw [15]. 09 rats were divided into three groups: the first group was used for the control treated with saline (0.9% NaCl). The second was treated with essential oil of *S. alba* (250 μl / kg), and the last group was treated with Ibuprofen at 10 mg/kg (250 μl / kg). The different treatments were administered intraperitoneally one hour before the formalin injection. After one hour of the treatment, the rats received 0.1 ml of a 1% formalin solution in the sub-plantar region of the right paw. The thickness of the paw was measured before 1, 2, and 3 h after the injection of formalin using a caliper. The edema volume was determined and expressed as a percentage of swelling based on the initial volume of the paw of each rat. Then, the rate of the inhibition was calculated according to the following formula:

$$Percentageinhibition(\%) = \frac{DC - DT \times 100}{DC} \qquad (2)$$

DC: Diameter of the control, D.T.: Treated paw diameter

2.9 Statistical analysis)

All the experiments were conducted in triplicate. Data are expressed as mean ± S.D. The data obtained were evaluated by one-way analysis of variance (ANOVA) with a $P < 0.05$.

3. Results

3.1 GC-MS analysis

The extraction gave us a high essential oil yield (2.35 ± 0.03%). The extraction yield of essential oil was influenced by several factors, such as the nature of the soil, the apparatus used, the operating pressure, the regularity of the temperature, the method, and the duration of distillation [16,17]. GC/MS was used to analyze the volatile components of *S. alba* in this study. Figure 1 showed the G.C. of the E.O. extracted from *S. alba*. The chemical constituents of E.O. were displayed in Table 1. The main components isolated from E.O. of *S. alba* were 3-Methylpentane (4.57%), 1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane (Eucalyptol) (2.93%), E-2-methylbut-2-enal (2.50%), and butyl 2-butoxy-2- hydroxyacetate (1.48%) (Table 1, Figure 1).

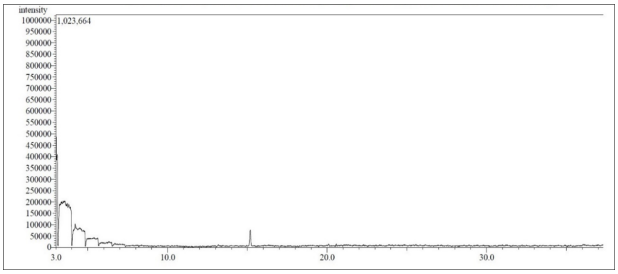

Figure 1. Chemical composition of essential oil of *S. alba*

S. alba essential oil was composed exclusively of monoterpenes, and the main identified compounds were eucalyptol (1,8 cineol) (2.93%). It exhibits anti-inflammatory action by inhibiting the production of arachidonic acid metabolites (leukotriene B4 and prostaglandin E2) and monocytes [18]. It has an inhibitory effect on the production of cytokines responsible for bronchial inflammation, stimulated by LPS and IL1beta by human monocytes in vitro (TNF-alpha, interleukin-1beta, leukotriene B4, thromboxane B2) [19]. It also has an analgesic and anti-inflammatory action through a non-opioid effect and its action on mast cells. Eucalyptol inhibits the production of TNF-α; it is a promising agent in the fight against pathologies associated with septic shock [20]. The GC-MS analysis revealed the presence of 3-Methylpentane (4.57%), a secondary metabolite recognized to present beneficial effects as antioxidant and anti-inflammatory compounds. 7-Oxabicyclo [4.1.0] Heptan-2-Ol represented a rate of 0.51% which was an epoxy-ionone derivative. It was a cyclic terpenoid; some of its derivatives occurred in plants, fruits, vegetables, and grains containing ß-carotene.

Previous studies have demonstrated that these compounds can exhibit significant pharmacological activities such as anti-inflammatory and anticancer [21]. The GC-MS analysis indicated the presence of Butyl 2-butoxy-2-hydroxyacetate with a rate of 1.48%. These results agreed with those described by [22], which identified the ester of acetic acid as a major component in Salicaceae essential oils. It was noted the presence of 2-[(5-Iodosalicylidene) hydrazino]-4-morpholino-6-(1-pyrrolidinyl)-1,3,5-triazine as derivative of Salicin. [23] indicated that a salicin derivative of willow extract prompted antioxidant enzymes and reduced oxidative stress by activating nuclear factor

Table 1. Chemical composition of essential oil of S. alba.

Peak#	R. Time	I. Time	Area%	Name
1	3.255	3.24	1.19	Oxirane, [(2-Propenyloxy) Methyl]-
2	3.278	3.265	1.44	Pentane, 3-Methyl-
3	3.353	3.33	2.5	E-2-methylbut-2-enal
4	3.395	3.38	1.48	Butyl 2-butoxy-2-hydroxyacetate
5	3.441	3.41	2.52	Pentane, 3-Methyl-
6	3.47	3.46	1	Pentane, 3-Methyl-
7	3.49	3.48	1.27	Heptane, 2,2,4,6,6-Pentamethyl-
8	3.516	3.505	1.29	3-Methylpentane
9	3.54	3.53	1.27	Dibutyl Ester
10	3.76	3.75	0.91	3-Methylpentane
11	3.81	3.77	4.57	3-Methylpentane
12	4.096	3.995	1.42	Butanal, 2-Methyl-
13	4.202	4.11	2.93	1,3,3-triméthyl-2-oxabicyclo[2,2,2]octane (Eucalyptol)
14	4.406	4.39	0.59	10-Bromodecyl Heptafluoro-2-Propyl Ether
15	4.755	4.74	0.51	7-Oxabicyclo[4.1.0]Heptan-2-Ol
16	5.031	4.99	0.66	4-Piperidinol, 2,2,6,6-Tetramethyl-1-Nitroso-
17	5.325	5.305	0.6	4-Amino-N'-[(2,4-Dichloro-5-Nitrophenyl) Methylidene]-1,2,5-Oxadiazole-3-Carbohyd
18	5.53	5.47	0.64	Stannane, Dichlorobis(Phenylmethyl)-
19	5.871	5.855	0.25	4-[(2,5-Dimethyl-3-Thienyl)Sulfonyl]Morpholine
20	6.111	6.07	0.21	1-Acetylmorpholine
21	6.288	6.265	0.28	5,8-Dimethoxy-6-[4-diethylamino-1-methylbutylamino] quinazoline
22	6.625	6.61	0.1	Carteolol, acetate (ester)
23	7.155	7.035	0.34	Pentanoic acid, 2-methyl propyl ester
24	7.21	7.17	0.17	Piperazine
25	8.125	8.09	0.05	Silane, [[(3.beta.,5.alpha.)-cholestan-3-yl]oxy] dimethyl (pentafluorophenyl)-
26	15.18	15.09	1.44	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1R)-
27	20.1	20.01	0.27	Verbenol
28	29.339	29.325	0.04	Sopharanol
29	32.23	32.175	0.13	3-[3-Chlorophenyl]-6-chloro-2(H)-quinolinone
30	32.595	32.56	0.06	2-[(5-Iodosalicylidene) hydrazino]-4-morpholino-6-(1-pyrrolidinyl)-1,3,5-triazine
31	37.455	37.39	0.03	Retinol

erythroid-2 related factor-2 in human umbilical vein endothelial cells. Variations in chemical composition are due to different environmental agronomic factors. Age and climatic factors also affect essential oil's chemical composition [24].

3.2 DPPH free radical scavenging assay

The results showed that the free radical's inhibition percentage has a proportional relationship with the concentration of *S. alba* essential oil. Thus, the DPPH inhibition levels recorded in the presence of the different essential oil concentrations of *S. alba* appeared to be greater than those of ascorbic acid (Figure 2). Thus, the essential oil of *S. alba* appeared to have better antioxidant activity than ascorbic acid ($P < 0.05$). This was probably related to the complexity of the essential oil in bioactive substances and even the synergy between them for better antioxidant activity [25,26].

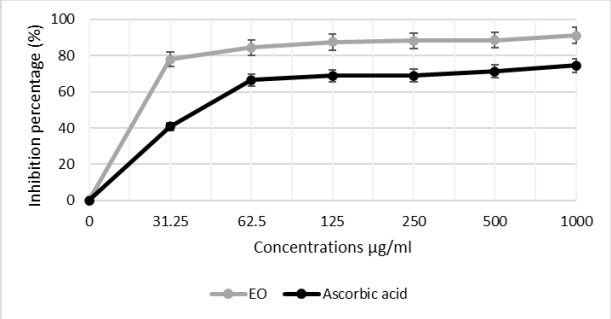

Figure 2. Inhibition Percentage (%) of *S. alba* E.O. and ascorbic acid.

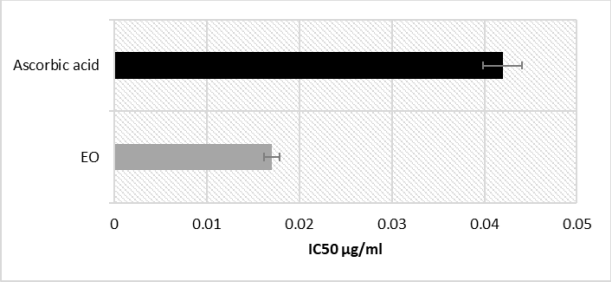

Figure 3. IC_{50} of *S. alba* EO and ascorbic acid.

The concentration of the essential oil necessary to inhibit 50% of the radical DPPH was calculated by linear regression of the percentages of inhibition calculated according to different concentrations of essential oil. The IC_{50} values for E.O. of *S. alba* and ascorbic acid are shown in Figure 3. The essential oil of *Salix alba* revealed interesting antiradical properties, manifested by a low value of IC_{50} (0.017 mg/ml). The comparison with ascorbic acid showed that *S. alba* E.O. has a higher antioxidant capacity than ascorbic acid, which has a substantially higher IC_{50} value (0.042 mg/ml). This was in perfect agreement with the results of [27], which showed a high antioxidant activity of the *Salix* genus with an IC_{50} value of 0.019 mg/ml.

S. alba leaves contain very active antioxidant substances, like phenols, that can prevent oxidation and reduce even the peroxides and oxidized substances. A study carried out on leaves of the genus *Salix* made it possible to identify phenolic compounds such as gallic acid, caffeic acid, vanillin, p-coumaric acid, myricetin, catechin, epigallocatechin gallate, and quercetin as well as salicin. This indicated the presence of high levels of phenols, volatile compounds, and flavonoids in *Salix* leaves, which can be used as a source of antioxidant compounds [27].

Various in *vitro* studies have shown the antioxidant activity of willow extract [28,29]. It was reported that in human vascular endothelial cells, willow bark extract could induce antioxidant enzymes and prevent oxidative stress by activating nuclear factor erythroid 2-related factor 2 (Nrf2) [30]. In addition to antioxidative and anti-inflammatory properties, willow bark is used in weight loss supplements [31]. Also, willow bark contains various flavonoids and polyphenols that synergistically contribute to the beneficial effects of *S. alba* [32,33].

3.3 Analgesic activity (hot plate method)

In this experiment, the rat's response to the hot plate's temperature set at 55 °C was recorded in a well-defined time interval (30, 60, 90 min). The response was expressed by the latency measured in seconds (Figure 4). After 30 min, it was found that rats treated with Tramadol had a time of 3.33 ± 0.6 sec for the reaction. Then the rat treated with S. alba E.O. has a 6 sec time to jump. At 60 min, the rats have a time of 5.66 ± 1.66 and 6.66 ± 0.83 sec for the Tramadol group and the essential oil, respectively. These results led us to conclude that *S. alba* E.O. exhibited resistance to pain caused by plate temperature compared to Tramadol ($P < 0.05$). This resistance showed the analgesic potential of S. alba E.O. Studies by [34] on *S. alba* showed that the bark of *S. alba* has both analgesic and inflammatory effects in albino mice, while phytochemical screening has demonstrated the presence of alkaloids, tannins, and glycosides.

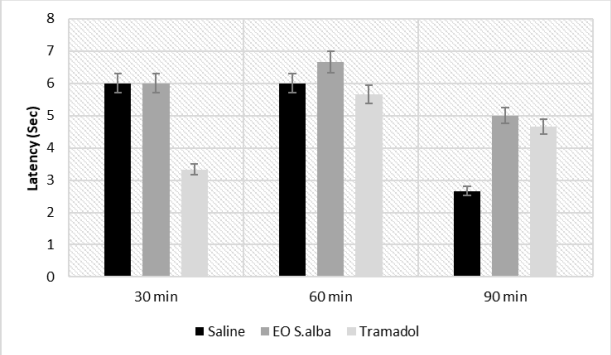

Figure 4. Effect of *S. alba* E.O. and Tramadol on rat response to plate temperature.

3.4 Analgesic activity (writhing test)

The injection of acetic acid 1.2% caused a painful symptom. The mechanism of occurrence of pain was the result of a tissue lesion responsible for an increase in the release of many chemical mediators, such as histamine, prostaglandin, and serotonin, in the intraperitoneal fluid, which will stimulate nociceptive receptors located at the peritoneal zone [35]. The pain was manifested as abdominal cramps (writhing). The contortion count was counted for each rat for 10 minutes. The results are shown in Figure 5.

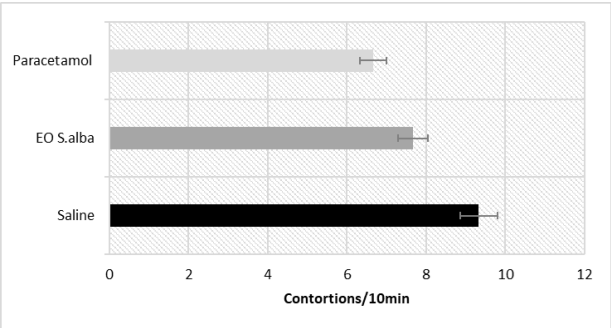

Figure 5. Analgesic effect of Paracetamol and S. alba E.O. against abdominal cramps.

These results found that acetic acid-induced an average of 9.33 ± 1.66 cramps recorded after 10 minutes in the control group treated with saline solution. Then, the other treatments showed several contortions equal to 7.66 ± 2.33 for the E.O. of *S. alba* at the dose of 250 μl / kg and 6.66 ± 2.16 for Paracetamol ($P < 0.05$). The inhibition percentage calculation recorded 17.89% and 28.61% values for the E.O. of *S. alba* and Paracetamol, respectively. This inhibitory action of *S. alba* E.O. was due to the presence of bioactive components that could inhibit the release of chemical mediators (such as histamine), which were responsible for the onset of the painful symptom.

3.5 Anti-inflammatory activity (the formalin test)

After 1 hour of formalin injection, an increase in the paw volume of the rats is noted with values of 5.82 ± 0.14 mm, 5.63 ± 0.18 mm, 5.24 ± 0.24 mm. for the group treated with saline solution, E.O. of *S. alba* and ibuprofen respectively ($P < 0.05$) (Figure 6). The action of inflammatory mediators expresses this increase. Under experimental conditions, formalin caused edema as local inflammation when injected into the sub-plantar part of the paw [36,37]. The cause of this inflammatory reaction is a tissue injury that induces the synthesis of histamine, prostaglandins, leukotrienes [38], PAF (platelet-activating factor), cytokines, NO (nitric oxide) and TNF (tumor necrosis factor) [39].

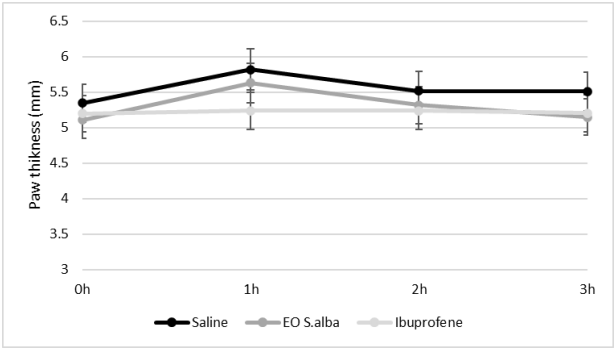

Figure 6. Evolution of the paw thickness.

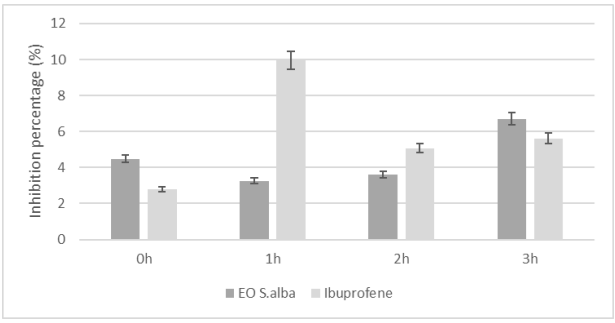

Figure 7. Inhibition Percentage of paw swelling in rats.

From 0-1.5 h, the inflammatory mediator is a prostaglandin (especially PGE2) (due to induction of COX-2), which coincides with thromboxane B2 [40]. Injection of the 1% formalin solution causes a biphasic response. The first phase is initiated immediately after the injection of the formalin solution and is characterized by stimulation of the C-fibers and release of the substance P and bradykinin. The second phase is due to local inflammatory pain [41]. During the first phase of inflammation (0-15 min), there was a small percentage of edema inhibition for *S. alba* E.O. and Ibuprofen, which was 4.49% for *S. alba* E.O. and 2.8% for Ibuprofen. Inhibition of edema by *S. alba* E.O. was less effective than those with Ibuprofen over time (Figure 7).

However, the drug used as a reference, Ibuprofen, was more effective in preventing these effects. Non-steroidal anti-inflammatory drugs that inhibit cyclooxygenase (COX-1 and COX-2) can effectively relieve acute pain [42]. After three h of treatment with *S. alba* E.O. and Ibuprofen, the results showed a recovery of the thickness of the paw with a value of 5.14 ± 0.01 mm for the E.O. of *S. alba* and 5.20 ± 0.05 mm for the drug Ibuprofen. This action is explained by the reduction of edema at the paw level. Inhibition of inflammation occurred during the time intervals of the experiment. All treatments produced reductions in inflammation ranging from 4.49 to 6.71% for *S. alba* E.O. and 2.8 to 5.62% for Ibuprofen (Figure 7). During the second phase (starting at 60 min), it was observed a significant percentage of inhibition of edema ($P < 0.05$) reached after three h treatment, a value of 6.71% and 5.62% for the E.O. of *S. alba* and Ibuprofen, respectively. However, it is also significantly lower than that determined by [43]. The study by [43] on the species *S. tetrasperma* and *S. subserrata* showed that the inhibition percentage of edema measured after three h of treatment with the extract of these two species is equal to 38% for *S. tetrasperma* and 29% for *S. subserrata*. The inhibition of the synthesis of these pro-inflammatory substances explains the effect of *S. alba* E.O. on edema. [44] investigated white willow bark extract's in vivo anti-inflammatory activity in a rat model of acute and chronic inflammation. The extract effectively reduced inflammatory exudates inhibited leukocyte infiltration, and prevented the increase of cytokines and prostaglandin. The extract was even more potent in inhibiting the COX-2 response than acetylsalicylic acid. Preliminary research suggests that willow bark extracts have analgesic, anti-inflammatory, and antipyretic effects [45]. Evidence demonstrates that willow bark extract providing 120-240 mg of the salicin constituent daily can reduce low back pain in some patients, with the higher concentration being more effective. Salicin's therapeutic effect had been reported to be comparable to Rofecoxib for low back pain [46].

4. Conclusion

The study showed that the essential oil of *S. alba* has interesting antiradical potential compared to the standard antioxidant (ascorbic acid). As for the analgesic potential, the essential oil of *Salix alba* showed resistance to temperature and even to the pain caused by the effect of acetic acid. Thus, the essential oil of *Salix alba* showed a reduction of inflammatory reaction in the induction model by formalin with different degrees of inhibition.

5. Conflict of interests

The authors declare that there is no conflict of interest

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